Functional size of acyl coenzyme A:diacylglycerol acyltransferase by radiation inactivation

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Abstract Rat liver acyl coenzyme A:diacylglycerol acyltransferase, an intrinsic membrane activity associated with the endoplasmic reticulum, catalyzes the terminal and rate-limiting step in triglyceride synthesis. This enzyme has never been purified nor has its gene been isolated. Inactivation by ionizing radiation and target analysis were used to determine its functional size in situ. ■ Monoexponential radiation inactivation curves were obtained which indicated that a single-sized unit of 72 ± 4 kDa is required for expression of activity. The size corresponds only to the protein portion of the target and may represent one or several polypeptides. —**Ozasa, S., E. S. Kempner, and S. K. Erickson.** Functional size of acyl coenzyme A:diacylglycerol acyltransferase by radiation inactivation. *J. Lipid Res.* 1989. **30:** 1759–1762.

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The enzyme, acyl coenzyme A:diacylglycerol acyltransferase (EC 2.3.1.20, DGAT), catalyzes the terminal step in triacylglycerol synthesis (1). In the liver this enzyme plays an important role in fatty acid and lipoprotein metabolism (1, 2). It is the only enzyme in the acylglycerol pathway that is committed exclusively to the formation of triglycerides. Its substrate, diacylglycerol, is an important branch point in acylglycerol synthesis because it can be converted either to triglycerides or to phospholipids. The active site of DGAT is localized to the cytosolic face of the endoplasmic reticulum (3). Diacylglycerol acyltransferase activity in isolated hepatocytes can be regulated by glucagon (4) and by exposure to fatty acids (2). The activity has been reported to be altered in vitro by phosphorylation–dephosphorylation (5).

Separate monoacylglycerol and diacylglycerol acyltransferases exist in rat liver (6). Although DGAT has been solubilized and partially purifed (6, 7), it has not yet been purified to homogeneity from any source, nor has its corresponding gene been isolated. Therefore, the required mass for expression of DGAT activity is unknown.

Many biochemical activities associated with membranes have proven intractable to purification. Thus, information about the nature of the molecular structures involved is limited. The technique of radiation inactivation can permit analysis of the functional size required for any given activity without the necessity of its prior purification. In the past, this technique has been used successfully to study in situ a number of intrinsic membrane enzymes localized to the endoplasmic reticulum (8–10) or to the plasma membrane (11). The main requirement for success of this approach is that the activity be stable to freezing and thawing of the membranes.

Exposing frozen biological samples to high energy electrons or gamma rays leads to gross destruction of molecules directly affected by the radiation, with consequent loss of associated biochemical activity. Because the interactions occur randomly throughout the mass of the irradiated sample, any measured activity decreases exponentially with increasing radiation exposure. The rate of loss is directly related to the mass of those molecules that participate in the activity or function measured. Simple exponential loss of function after increasing doses of radiation permits straightforward application of target analysis. The target sizes determined in this way represent a radiation-sensitive macromolecular assembly that contains the biochemically active structure(s) of interest. In the simplest case, the target size corresponds directly to the active unit. Target analysis has also been developed for more complex systems (12, 13).

MATERIALS AND METHODS

Male Sprague-Dawley rats, 180–200 g (Simonsen, Gilroy, CA) were housed individually under reversed

Abbreviation: DGAT acyl coenzyme A:diacylglycerol acyltransferase.
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lighting (lights on 6:00 PM, lights off 6:00 AM). They were maintained on Purina rat chow and water ad libitum. At D6 (mid dark phase), microsomes were prepared from pooled livers of three rats as described previously (14). The microsomes were resuspended in buffer containing 0.1 M sucrose, 0.05 M KCl, 0.03 M EDTA, 0.04 M KH_2PO_4, pH 7.2, at approximately 10 mg protein/ml, and glucose-6-phosphate dehydrogenase ((EC 1.1.1.49), bakers yeast, Type VII, Sigma, St. Louis MO) was added (15). Samples were aliquoted at 0.5 ml/vial into 2-ml glass vials; the vials were frozen at −70°C, and sealed with an oxygen-gas flame. Samples were kept at −70°C except during radiation exposure which was performed at −135°C. High energy electrons were used to deliver predetermined doses of radiation to the samples as described previously (8).

Glucose-6-phosphate dehydrogenase was assayed spectrophotometrically as described previously (8). Glucose-6-phosphatase (EC 3.1.3.9) was assayed according to de Duve et al. (16). Acyl coenzyme A:diacylglycerol acyltransferase was determined by measuring incorporation of [^{14}C]oleate from [^{14}C]oleoyl CoA into triglyceride as described previously (17). This assay is similar to that described by Haagsman and Van Golde (2).

All assays were in the linear range with respect to protein concentration, substrate, and time of assay under the conditions used (data not shown). There was no detectable effect of freezing and thawing the microsomes on the activities of glucose-6-phosphatase or DGAT. All assays were run in triplicate. Measurements of enzyme activity in irradiated samples were normalized to the activity determined in unirradiated samples. Data were analyzed by a least squares analysis constrained to 1.0 at zero radiation dose. Target sizes, calculated as described (18), are given as the average ± SD from three or more independent experiments.

RESULTS AND DISCUSSION

In order to validate the techniques and procedures used in this study, two other enzymes were examined in the same irradiated samples as those used for assay of DGAT.

Pure glucose-6-phosphate dehydrogenase was added to the microsomes during sample preparation as an internal standard. The target size obtained for glucose-6-phosphate dehydrogenase was 114 ± 6 kDa, in agreement with previously published values (8, 15), indicating that the experimental conditions had not introduced radiation artifacts.

Glucose-6-phosphatase is intrinsic to the liver endoplasmic reticulum. It was used as a membrane-associated internal standard. Although glucose-6-phosphatase

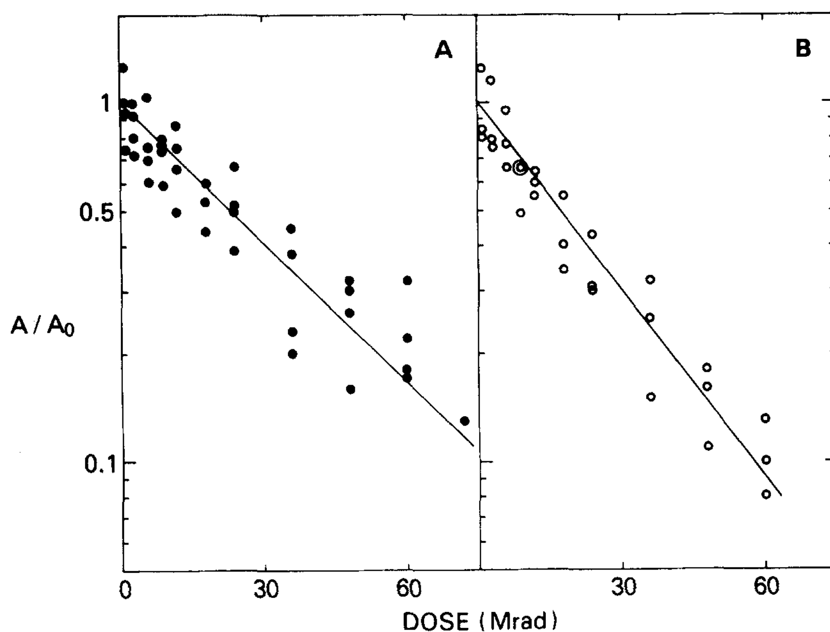

Fig. 1. Loss of enzymatic activity from frozen rat liver microsomes as a function of radiation dose. Liver microsomes were prepared and assayed as described in Methods. Four independent experiments were performed for the analysis of glucose-6-phosphatase (panel A) while results from three independent experiments are shown for the analysis of triglyceride synthesis (panel B). For each experiment, microsomes were prepared from livers pooled from three rats. Initial glucose-6-phosphatase activity was 5.15 ± 2.20 μg P_i/min per mg protein. Initial diacylglycerol acyltransferase activity was 0.148 ± 0.016 nmol triglyceride/min per mg protein. Each point is the average of triplicate determinations.

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has not yet been purified, its size has been determined previously by radiation inactivation (19, 20).

In our preparations, glucose-6-phosphatase activity decreased monoexponentially with radiation dose (**Fig. 1A**), yielding an average target size of 54 ± 8 kDa from four independent experiments. This value was smaller than the target size for this enzyme of 70 kDa reported by Collipp et al. (19) using radiation inactivation analysis of lyophilized human or rat liver microsomes. Irradiation of lyophilized membranes can result both in increased scatter among the experimental data and in aberrantly large target sizes (11). Our value, obtained under experimental conditions designed to minimize artifacts, should more nearly reflect the true mass required for activity and agrees well with that reported by Ness et al. (20), 53 ± 18 kDa, assayed under conditions similar to ours. The apparently normal behavior of glucose-6-phosphatase in our preparations after radiation inactivation suggests that no general membrane-associated artifacts had been induced by radiation.

Triglyceride synthesis was measured in situ by the incorporation of [^{14}C]oleoyl CoA and endogenous diacylglycerol into triglycerides and thus predominantly reflects DGAT activity. Radiation inactivation curves for DGAT were monoexponential and indicated that a single-sized unit of 80 ± 16 kDa was required. This large error is due principally to the results from one experiment that were inconsistent with the other three; deleting this experiment results in an average target size of 72 ± 4 kDa (Fig. 1B). The target size of 72 kDa reported here corresponds to the minimal size of the molecular structure needed for the synthesis of triglycerides in intact rat liver microsomes. This is the first estimate for the size of this enzyme. The target may be one or several polypeptides, and if it is a glycoprotein, represents only the protein portion of the molecule (21).

Most common biochemical techniques to determine the size of membrane proteins require solubilization. However, reagents which dissociate enzymes from membranes may denature the native conformation, abolish activity, separate noncovalently linked subunits necessary for activity, or lead to proteolytic clipping of the protein. In such cases it may never by possible to obtain the enzyme in its native active form. One of the great advantages of the radiation inactivation technique is that a function can be studied in situ. Thus, the determination by radiation inactivation of the functional size of the intrinsic membrane activity, acyl coenzyme A:diacylglycerol acyltransferase, provides an estimate of the size of the structure required for expression of this enzyme activity in the intact cell. ■

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